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Genetic Manipulation of Starch Biosynthesis: Progress and Potential

H. FRANCES J. BLIGH

Division of Plant Science, School of Biological Sciences, University of Nottingham, Nottingham, NG7 2RD, UK

Introduction

Of the starch produced commercially in the western world, approximately a third is used for non-food applications, while the remaining two thirds is used in the food industry. The use of starches in non-food applications is one which is becoming of increasing importance also for consumers as they become more aware of 'green' issues such as sustainability of raw material sources and biodegradation of materials (Jane *et al.*, 1994b), especially packaging. The need of industry for starches to produce, for example, plastics, packaging and paints, has resulted in research targeted towards producing starches with specific properties required for these specialist applications, as well as towards increasing the yields of starch producing plants. In addition to non-food uses, starch is used widely in the food industry, and, with the development of more processed and pre-prepared foods, demand for specialist starches is increasing. The majority of commercial starch is sourced from potato, maize and wheat, and as a result, these are the systems used most in studies of starch biosynthesis and structure, with most emphasis being placed on potato and maize. However other organisms have also been used, notably rice, which as a simple monocotyledonous plant is a useful model for the hexaploid wheat, and pea, which like maize, exhibits useful visible starch mutant phenotypes. Barley has also been used, as there is commercial interest in this as a source of starch in countries which do not have suitable growing conditions for crops such as potatoes or maize. More recently, intensive studies have been performed on crops such as cassava, which is widely grown in tropical climates, with a view to genetic improvement (Munyikwa *et al.*, 1997).

Starch structure and synthesis

Starch is a major storage carbohydrate found in photosynthetic and storage organs of plants. As starches for industrial use come from storage organs, mainly seeds and

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tubers, this review will deal with synthesis in these organs only. Starch in storage organs is synthesized within amyloplasts, plastids which can contain either a single large starch granule, as in potato, or multiple smaller granules as in rice. The granules themselves can also vary with respect to shape and structure. Rice has polyhedral starch granules which are between 3 and 8 µm in diameter, while potato has ovoid granules which can be up to 100 µm in diameter (Jane *et al.*, 1994a) (*Table 1*). Despite these differences, microscopic analysis of granule structure has shown some features which appear to be constant. All granules have been shown to exhibit a concentric sphere morphology, with alternating semi-crystalline and amorphous growth shells. Growth of these rings was thought to be linked to light and dark diurnal cycling, but is now thought to be due to day to night temperature variation (Buléon, 1997). Analysis of the semi-crystalline shells has been shown to contain further stacks of alternating crystalline and amorphous lamellae (Jenkins *et al.*, 1993). The repeat size of one layer of crystalline and amorphous lamellae appears to be approximately 9 nm, irrespective of botanical origin. However, the proportional size of crystalline and amorphous layer within the 9 nm layer has been shown to vary according to the molecular composition of the starch (Jenkins and Donald, 1995). More recently, further levels of complexity have been demonstrated showing the amylopectin lamellae to be organized into blocklets (Gallant *et al.*, 1997) (see *Figure 1*).

Table 1. Average size and shape of starch granules from botanical sources

Botanical origin	Diameter range (microns)	Shape
Maize	5–20	spherical or polygonal
Potato	15–75	oval or spherical
Rice	3–8	polygonal
Wheat A type	22–36	disk
Wheat B type	2–3	spherical
Barley A type	10–48	oval
Barley B type	2–10	spherical
Pea	10–45	irregular with indentation
Cassava	5–40	rounded with indentation
Banana	15–45	irregular
Phaseolus	10–45	disc shaped
Maize *su* mutants	2–3	spherical

At the molecular level, starch is made up of two components, amylose, which consists of linear chains of α(1–4) linked glucose molecules, and amylopectin, which is made up of many shorter α(1–4) linked chains that also have branches in the form of α(1–6) linkages. The chains of amylopectin fall into several classes, A chains which have no substitution at the 6 position, B chains, which are branched at one or several points by α(1–6) linkages, and C chains, a single one of which runs through the centre of the amylopectin cluster and has a reducing end (Robin *et al.*, 1975) (*Figure 1*). The packing of A chains of the amylopectin are thought to be responsible for the type of crystallinity exhibited by the starch, as measured by X-ray diffraction. Shorter A chains are thought to be associated with A-type crystallinity, longer A chains associated with B-type crystallinity and C-type crystallinity being thought to be a result of a mixture of A and B types (Hizukuri, 1985). Crystallinity is also an indicator of the digestibility of a starch, with B and C-type starches, found in tubers and

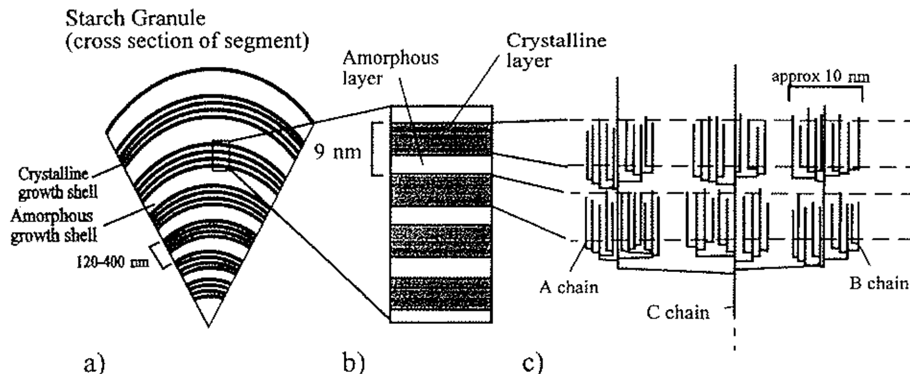

Figure 1. Schematic of starch granule structure. Cross section through a section of starch granule showing semi-crystalline and amorphous growth shells. Magnified view through one semi-crystalline shell showing amorphous and crystalline lamellae. Fine structure of crystalline and amorphous lamellae showing A, B and C chains lining up in the crystalline lamellae in blocklets with branchpoints within the amorphous layer. Based on Jenkins and Donald (1995) and Gallant *et al.* (1997).

legumes, being associated with poorer digestibility and higher enzyme resistance than A-type starches, found in cereals (Gallant *et al.*, 1972). The enzyme resistance aspect of starches is particularly relevant in the food industry where a lot of starch is processed to produce maltodextrins and corn syrup. It is also the amylopectin that provides the viscosity of starch that is required for its many food uses in applications such as thickenings and pastes. The percentages of amylose and amylopectin vary from plant to plant, and also within varieties of a plant species. For any major progress to be made in the manipulation of either yield or quality of starches within the plant, as opposed to production via post harvest modification, the fundamental processes involved in the biosynthesis of starch in storage organs, such as seeds and tubers, must be understood. So far, the enzymes involved in starch biosynthesis within the amyloplast fall into three main groups (see *Figure 2*) and a large number have now been cloned from various plant sources (*Table 2*). The first group is the ADP glucose pyrophosphorylases (AGPase), which are responsible for determining the flux of carbon into the starch biosynthesis pathway by synthesizing ADP-glucose, the substrate for starch synthases, from ATP and glucose-1-phosphate. The starch synthases transfer glucose from ADP-glucose substrate to α(1–4) glucan to create amylose and fall into two groups, Granule Bound Starch Synthases (GBSS) and Soluble Starch Synthases (SSS). The GBSSs are involved in the synthesis of amylose, as has been shown by the fact that null mutants of these genes, first isolated as *waxy* mutants in maize, are almost totally lacking in amylose. The SSSs are thought, by default, to be involved in the synthesis of the glucose chains that act as substrates for the Branching Enzymes, although there is little evidence to confirm this. The Branching Enzymes (BE) create the α(1–6) branches of the amylopectin and appear to fall into 2 groups, A and B, based on sequence homology. Mutations in some A group BE genes appear to result in what is known as the *amylose extender (ae)* mutant, which is characterized by particularly long chain amylopectin, giving the characteristics of a high amylose starch. This led to the theory that the B group of BEs had affinity for branching long glucose chains, while the A group had affinity for shorter chains, however, this

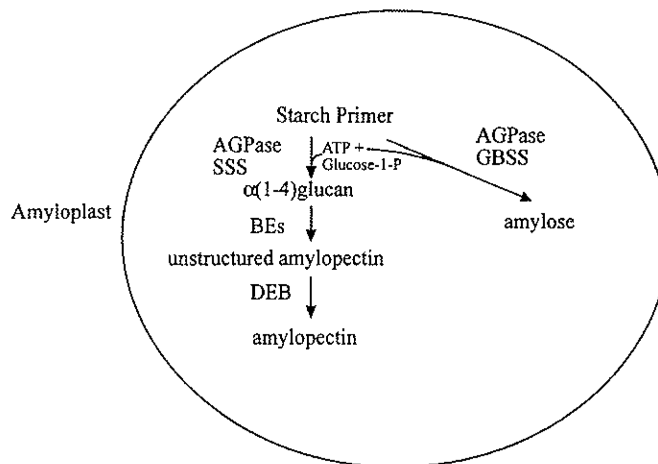

Figure 2. Schematic of enzyme groups responsible for starch biosynthesis. ATP and glucose-1-phosphate are converted to ADP glucose by ADP glucose pyrophosphorylase (AGPase), which is then used as a substrate by soluble starch synthase (SSS) or granule bound starch synthase (GBSS) to make glucan chains. Unstructured amylopectin is then thought to be created by branching enzymes (BE) and then broken down to an ordered structure by debranching enzymes (DBE).

mechanism is probably an oversimplification of a more complex interaction between both branching enzymes and starch synthases. In addition to these three main groups, there are also debranching enzymes (DBE), pullulanase and isoamylase which were initially thought to be involved in degrading of starch during germination (for review see Nakamura, 1996). Recent expression studies have suggested that these enzymes are involved more directly in starch synthesis with DBEs thought likely to be mandatory in creating the rigid structure that results in the concentric shell formation seen in starch granules (James *et al.*, 1995; Ball *et al.*, 1996). Isoamylase is also now thought to have an important role in amylopectin structure, as the *sugary-1* (*su-1*) mutant of maize, a mutant with increased phytoglycogen and amylose, has now been identified as a gene with strong homology to bacterial isoamylase enzymes (James *et al.*, 1995). The importance of the two different forms of DBE, pullulanase and isoamylase, may vary between plants, as *su-1* mutants of rice have been demonstrated to have significantly low levels of pullulanase but no discernable difference in isoamylase expression (Nakamura *et al.*, 1997). Disproportionating enzyme, so far found only in potato, is another enzyme also thought initially to be involved in starch degradation that has now been shown to be highly expressed during tuber development (Takaha *et al.*, 1993). As this enzyme is thought to be able to use short chain amylose and amylopectin as substrate, it may be that this enzyme has a joint role with debranching enzymes in regulating the number of branches and chain length of amylopectin. There is also a need for a primer molecule to initiate the starch synthesis. Such a primer, named amylogenin and thought to be functionally analogous to glycogenin, was identified by Keeling *et al.* (1994) although recent data has led to debate as to whether this is the true function of this enzyme (Dhugga *et al.*, 1997). The need to understand the interactions and mode of action of enzymes involved in starch biosynthesis has been the driving force behind a lot of the

Table 2. Genes from major starch sources involved in starch biosynthesis that have been cloned

Enzyme	Source of Clones	References
AGPase small subunit	rice, potato, maize, wheat, barley	Anderson *et al.* (1989); Smith-White and Preiss (1992); Bhave *et al.* (1990); Olive *et al.* (1989); Villand *et al.* (1992)
AGPase large subunit	rice, potato, maize, wheat, barley	Satozawa *et al.* (1995); Nakata *et al.* (1991); Giroux *et al.* (1995); Olive *et al.* (1989); Villand *et al.* (1992)
GBSS	rice, potato, maize, wheat, pea, barley	Wang *et al.* (1990); Visser *et al.* (1989); Klösgen *et al* (1986); Clarke *et al.* (1991); Dry *et al.* (1992); Rohde *et al.* (1988)
SSS	rice, potato	Baba *et al.* (1993); Marshall *et al.* (1996)
Branching enzyme type A	rice, maize, pea	Mizuno *et al.* (1993); Fisher *et al.* (1993); Burton *et al.* (1995)
Branching enzyme type B	rice, maize, potato, pea	Nakamura and Yamanouchi (1992); Fisher *et al.* (1995); Poulsen and Kreiberg (1993); Burton *et al.* (1995)
Debranching enzyme (pullulanase)	rice	Nakamura *et al.* (1996)
Debranching enzyme (isoamylase)	maize	James *et al.* (1995)
Disproportionating enzyme	potato	Takaha *et al.* (1993)

experiments performed to date involving the manipulation of starch biosynthesis enzymes.

Methods for genetic manipulation

Genetic manipulation of starch falls into two main categories. The first is the more obvious use of transgenic gene expression using modern molecular biology techniques to increase or reduce the expression of specific enzymes. In common with all transgenic expression systems, there are certain requirements:

(a) The availability of a reliable transformation system for the target organism. Until recently, this has been a particular problem for cereals such as wheat and rice, although biolistics, and latterly, the development of supervirulent *Agrobacterium* strains have both played a role in the increase in the number of species of cereals that can now be transformed reliably.

(b) A highly expressing promoter system. A strong promoter is essential if a transgene is to be highly expressed, and while many model systems have successfully used the constitutive Cauliflower Mosaic Virus 35s rRNA promoter (Croy, 1993), this is not always highly expressed, and also has the disadvantage that it is expressed indiscriminately in all tissues. This problem was encountered by Stark *et al.* (1992) who expressed the *Escherichia coli* AGPase gene in potato, but found that while they could regenerate shoots and callus, the over-expression caused a lethal effect resulting in poor recovery of transgenic plants expressing the gene. This problem was overcome by using the tuber specific patatin promoter that targeted

the expression almost exclusively to the tuber, and enabled the regeneration of healthy plants. Seed specific promoters such as the rice glutelin (Leisy *et al.*, 1990) and the maize GBSS (Russell and Fromm, 1997) promoters are also now available and the increasing availability of such promoters means that ideally, a promoter can be chosen that will have optimal expression in the chosen plant system.

(c) Ideally, such promoters should also be accompanied by a terminator sequence, as while not essential, these sequences have been shown to have some role in the expression and regulation of genes (Nakata and Okita, 1996). In addition to this, it is necessary to examine the expression systems of the plant in which expression is required. One example of this is in some cereals such as maize and rice, where it has been demonstrated that the presence of a large (>1kb) intron in the 5' untranslated region can lead to improved expression of transgenes (Maas *et al.*, 1991; Li *et al.*, 1995). For more comprehensive details about expression systems the reader is referred to Croy (1993).

There are three techniques that have the potential to be used in the molecular manipulation of starch enzymes.

1. The use of antisense technology to reduce, or completely inhibit a particular gene activity. This method constitutes taking the coding region of a gene, or a section of it, and placing it in the reverse orientation under the control of a suitable promoter. This is thought to result in the production of an 'antisense' mRNA molecule that is complementary to and hence can form a duplex with the mRNA of the gene whose suppression is desired. Similar effects to this have also been demonstrated when attempts have been made to over express an endogenous gene in plants. For some reason, it has been observed that over-expression of a gene can result in suppression of that gene in much the same way as antisensing can. This phenomenon has been termed sense suppression and is used with as much success as antisense suppression in the inhibition of gene activity. Both antisense and sense suppression have been used to study starch biosynthesis genes in potato (Müller-Röber *et al.*, 1992; Kuipers *et al.*, 1994; Flipse *et al.*, 1996a–d) and rice (Shimada *et al.*, 1993; Itoh *et al.*, 1997).
2. Over-expression of an endogenous gene. This method can be used to increase the expression of a gene either to increase the overall target product, such as over-expression of AGPase to increase starch production, or alternatively to increase a particular enzyme function such as BE, to increase the overall branching levels within the starch. Over-expression of endogenous genes to increase gene activity has met with little success due to the phenomenon of sense suppression. This appears to be caused by natural feedback mechanisms within the plant preventing genes from being expressed at higher levels than would be found naturally (see above) (e.g. Itoh *et al.*, 1997).
3. The third method by which starch may be modified transgenically is by the use of heterologous gene expression. This is where the transgene used is from a different genetic source from the system being transformed, for example, another plant or a completely different organism such as *E. coli*. This approach has been taken by several investigators expressing genes such as the *E. coli* AGPase or BE genes

(Stark *et al.*, 1992; Shewmaker *et al.*, 1994; Sweetlove *et al.*, 1996a,b; Kortstee *et al.*, 1996). In addition to the usual requirements for transgenic expression described above, bacterial transgene constructs have a few additional requirements for functional expression. A requirement for expression of starch enzymes in particular, is a targeting sequence to enable the expressed protein to target to the amyloplast. In the case of the *E. coli glg*C16 gene in potato, a chloroplast transit peptide from the *Arabidopsis thaliana* small subunit ribulose 1,5-bisphosphate carboxylase (Stark *et al.*, 1992). In addition to this, it is often necessary to check that the sequence environment around the start AUG codon is suitable for plant expression, as the sequence around the AUG has to have at least some homology to the consensus AACAAUGGC (Futterer and Hohn, 1996).

The second less obvious, but no less useful, method of manipulating gene expression, is the use of known mutants or alleles to breed for a particular starch characteristic (see *Table 3* for list of mutants). A major example of this is the use of *waxy* maize varieties that have been cultivated due to industrial uses of *waxy* maize starch. These varieties have also been used in breeding studies to produce novel starches such as the loosely branched starch obtained by Boyer *et al.* (1976) who crossed a *wx* maize with a variety carrying the *ae* mutation. Similar crosses with *wx* and *du* genes have been shown to result in an increase in shorter B chains of the amylopectin (Fuwa *et al.*, 1987). Mutagenesis followed by screening for starch mutants has also been employed more recently in peas by Hedley and colleagues who used both mutagenesis and breeding techniques to develop a series of novel starches with altered starch granule shape (Wang *et al.*, 1990, 1997; Lloyd *et al.*, 1996). Utilizing both the *rugosus* (*r* and *rb*) mutants of classical genetics and a series of others, a total of 6 alleles involved in starch biosynthesis were identified. By studying the physical nature of the starches in these mutants, combined with an analysis of starch biosynthesis enzyme activities, the enzymes associated with these loci were identified. Having identified the individual loci for these alleles, a series of further breeding experiments were performed to create double mutants, similar to those of Boyer *et al.* (1976) and Campbell (1996) for maize. The double mutants obtained had further modifications to both starch structure and content, including some interesting effects on starch granule morphology in the case of a *rb/rug-5* double mutant (Wang *et al.*, 1997). It is hoped that further study of such double mutants will extend our knowledge into the interactions of starch biosynthesis enzymes as well as allow for the breeding of novel starches for commercial use. These methods have previously been particularly slow and cumbersome, but the advent of modern genetic screening methods such as microsatellite DNA markers, has made targeted breeding programs considerably quicker and easier. A large number of the starch biosynthesis genes have now been mapped in both maize and rice, and in the case of rice, a microsatellite marker which is closely linked to the GBSS gene has been identified (Bligh *et al.*, 1995). This marker has been shown to be useful in identifying alleles of the GBSS gene which have intermediate amylose, and this in turn has the potential to be used as a breeding marker to select for intermediate or high amylose levels (Ayres *et al.*, 1997). Mapping of the BEIII gene of rice also enabled the prediction of the location of this gene in other cereals, as the maize BEII and rice BEIII genes, both thought to be group A branching enzymes, were found to be in regions within their respective genomes. These regions corresponded to homeologous regions

Table 3. Mutations known to affect structural genes for enzymes involved in starch biosynthesis

Mutation	Enzyme affected	Plant	Phenotype	Reference
waxy (wx)	GBSS	maize	no amylose	Shure *et al.* (1983)
waxy (wx)	GBSS	rice	no amylose	Sano (1984)
amylose free (amf)	GBSS	potato	no amylose	Hovenkamp-Hermelink *et al.* (1987)
low amylose (lam)	GBSS I	pea	low amylose	Denyer *et al.* (1995)
amylose extender (ae)	BE IIb	maize	high amylose	Fisher *et al.* (1996)
amylose extender (ae)	BE III	rice	high amylose	Mizuno *et al.* (1993)
brittle-2 (bt-2)	small subunit AGPase	maize	low starch	Giroux *et al.* (1994)
shrunken-2 (sh-2)	large subunit AGPase	maize	low starch	Giroux *et al.* (1994)
rugosus (r)	BE I	pea	low starch	Bhattacharya *et al.* (1990)
rugosus (rb)	small subunit AGPase	pea	low starch	Smith *et al.* (1988)
rugosus-5 (rug-5)	GBSS II	pea	altered starch granule shape	Wang *et al.* (1997)
sugary-1 (su-1)	isoamylase (debranching enzyme)	maize	high phytoglycogen	James *et al.* (1995)

in the oat and wheat genomes (Harrington *et al.*, 1997). The homeology of such genes within groups such as cereals has the potential to be utilized in breeding programs.

Targets for genetic manipulation

1. One of the major reasons for genetically manipulating starch biosynthesis enzymes is to increase our understanding of how the enzymes interact to produce starch. For many of the more targeted experiments designed specifically to construct an altered starch with a view to commercial exploitation, knowledge is often also gained regarding the mode of action of an enzyme, or its interaction with other enzymes in the pathway. Hence, this particular area, to some extent, is mixed in with experiments described in other sections below. One of the earliest starch gene antisense experiments was the antisensing of the potato small subunit AGPase gene. This resulted in a tuber with reduced dry weight, high sucrose (up to 30%) and reduced storage protein, thus demonstrating the crucial role of AGPase in overall starch biosynthesis (Müller-Röber *et al.*, 1992). In some cases, it is possible to understand the role of an enzyme because a null mutant of the enzyme has been isolated, e.g. the *waxy* mutant of maize has been demonstrated to have no, or little amylose, as this mutant is null for the GBSS. However, this is not always the case and some enzymes, such as the SSSs and some BEs have no known null mutants. An example of how transgenic plants can be used to study genes for which there are no known phenotypic mutations is in the antisensing of BE in an amylose free potato by Flipse *et al.* (1996d). This experiment would have been expected to result in the originally amylose free (*amf*) potato starch, which normally stains red with iodine, regaining at least some amylose, due to lack of branching resulting in longer glucose chains. In fact, although some of the smaller starch granules did stain blue at the core, no discernible change was found in the branching or starch levels of these potatoes compared with the controls. More interestingly, changes were found in the physico-chemical properties of this

starch, specifically in the rheological properties, which are known to be affected by amylose:amylopectin ratios, suggesting that suppression of the BE had resulted in subtle changes to the amylose levels. Despite the fact that only one gene had been isolated for BE in potato at the time, this evidence suggested that, in common with other plants, potato does indeed contain a second BE, which is responsible for the branching levels seen in the starch of these potatoes. This would appear to be confirmed by the work of Larsson *et al.* (1996) who detected a second BE isoform in potato starch, although the actual gene has still to be isolated. Another approach that has been taken is that of expressing plant starch biosynthesis enzymes in *E. coli* and observing how these enzymes affect the glycogen synthesis in this bacterium. *E. coli* itself has a well characterized glycogen synthesis pathway, and strains mutant for the three major genes of the pathway, the AGPase, glycogen synthase and the branching enzyme are all available (Damotte *et al.*, 1968; Cattanéo *et al.*, 1969). Expressing plant enzymes such as potato AGPase in *E. coli* gives us the opportunity to observe aspects of enzyme activity in a simple system which also enables large quantities of the enzyme to be cultivated for purification relatively rapidly. This system also enables assessment of enzyme activity away from isoforms and other enzymes, which may co-purify and affect results. Such a system has been used to great effect to study the allosteric regulation of potato AGPase subunits by Okita and others, who have expressed both the large and small subunits of this enzyme in *E. coli* to obtain a functional enzyme (Iglesias *et al.*, 1993). By first mutagenising potato large subunit cDNA and then expressing the potato large and small subunit genes in a *glgC*$^-$ strain, it was possible to identify mutants with impaired glycogen production. One particular mutant was identified as requiring higher levels of 3-phosphoglycerate for maximum activity and on sequencing, a single nucleotide change of Pro-52 to Leu was found (Greene *et al.*, 1996a). The same technique was used to identify Asp-413 as a key requirement for 3-phosphoglycerate binding in allosteric regulation of this enzyme as mutation of this residue to alanine resulted in almost complete abolition of activation by 3-phosphoglycerate (Greene *et al.*, 1996b). Similar systems have also been set up expressing maize BEI and II in *E. coli* (Guan *et al.*, 1994a,b; Guan *et al.*, 1995). The expression of both of these branching enzymes in isolation has enabled the substrate interactions for these enzymes to be studied in more detail. This has demonstrated that the purified enzymes do have specificity for different chain lengths, with BEI predominantly longer chains than BEII, as had been predicted from mutation studies (Guan *et al.*, 1997). This heterologous expression system has also enabled studies of predicted active sites, deduced from homologies between enzymes of different species, to be examined by deleting well conserved amino acids, and then assessing the activity of the enzyme. This demonstrated the importance of Asp-386, Glu-441 and Asp-509 residues, all of which were found to be essential for enzyme activity (Kuriki *et al.*, 1996). More interesting, from the point of manipulating starch structure, is the construction of BEI/BEII hybrid enzymes. While most chimeric molecules produced by this method resulted in loss of enzyme activity generally, one hybrid enzyme, which consisted of predominantly BEII, with only the carboxy-terminal part of BEI, showed a high activity with substrate affinity similar to that of BEI. This demonstrates the importance of this

region of the protein in substrate specificity and hence chain length in amylopectin (Kuriki *et al.*, 1997). In the case of both BE and AGPase, it is interesting to note that the plant enzymes will complement the equivalent *E. coli* mutations, despite the fact that *E. coli* synthesizes glycogen and not starch, indicating the strong similarities between starch and glycogen biosynthesis. A similar model system for examining starch biosynthesis enzymes at the more basic level is that of *Chlamydomonas reinhardtii*, a single celled alga that can both photosynthesize and synthesize starch. The starch of this alga has been shown to have A type crystallinity, the same as that found in maize, and studies have shown the two starches to be indistinguishable, making this an ideal model organism for starch biosynthesis studies (Buléon *et al.*, 1997). Using X-ray mutagenesis, it has been possible to create a large number of starch mutations that could be identified by staining with iodine vapours. Iodine naturally stains starch black, but mutants of *Chlamydomonas* could be identified easily as they stained either yellow, red, or olive, depending on the type of mutant starch present (Ball *et al.*, 1991; Fontaine *et al.*, 1993). Using this series of mutants, the effect of mutations on genes for which no known phenotypes exist in plants have been studied and alleles corresponding to most of the major plant starch synthesis enzymes have been identified. The *st-3* mutation, which lacks one isoform of soluble starch synthase (SSII), leads to an apparent increase in amylose. It also causes an increase in the number of short chain (approx. 6 residues) and decrease in the number of intermediate chain glucans (10–40 residues) in the amylopectin (Fontaine *et al.*, 1993). The *st-2* mutants deficient in GBSS, as well as being deficient in amylose, also had altered amylopectin (Maddelein *et al.*, 1994) with a decrease in extra long (greater than 50 residues) chains. These studies have led to the conclusion that each starch synthase interacts at a different level with each branching enzyme to create a varying degree of branching (Buléon *et al.*, 1997). The role of a starch synthase in the determination of amylopectin chain length would appear to correlate with the studies on potato, where expression of *E. coli* glycogen synthase resulted in the production of a highly branched amylopectin, not dissimilar to glycogen (Shewmaker *et al.*, 1994). Studies on the *st-7* mutant in *Chlamydomonas*, which lacks an isoamylase branching enzyme, have led to the current theory that amylopectin is formed from a preamylopectin precursor by processing by debranching enzyme (Mouille *et al.*, 1996). Another interesting finding that arose from this work is the discovery that *Chlamydomonas* starch granules do not contain the characteristic ring structure associated with day/night cycles in plants. It has been speculated that this is due to the growth of this organism under controlled conditions with constant temperature and light, and that such a system would be useful in the study of the growth and pattern of these rings (Buléon *et al.*, 1997). Such work is invaluable in laying groundwork on which theories can be based regarding how various plant enzymes may be manipulated in a controlled fashion to obtain altered starches. It remains to be seen how far such studies can be extrapolated to higher plants.

2. One of the major goals for manipulation of starch for industrial purposes is that of increasing the amount of starch synthesized in the storage organ. As the rate-limiting enzyme for starch synthesis is AGPase, this has obviously been the main target enzyme in these studies. Two groups have taken the route of over-

expressing the AGPase gene in potato (Stark *et al.*, 1992; Sweetlove *et al.*, 1996a,b). Due to the problems of sense suppression, allied with the fact that both phosphate and 3-phosphoglycerate allosterically regulate AGPase, both groups took the approach of using the *E. coli glg*C16 mutant. This has the double advantages of a) having very little homology with the plant's genes and so being unlikely to cause sense suppression, and b) the gene has a substitution of aspartic acid for glycine at position 336, which makes it resistant to allosteric inhibition (Leung *et al.*, 1986; Lee *et al.*, 1987). In both cases as well, the gene was expressed under the control of the patatin promoter and targeted to the amyloplast using the transit peptide sequence from a small subunit ribulose-bisphosphate carboxylase. Both groups claim to have increased the activity of AGPase in the transgenic potato tubers with Stark *et al.* (1992) claiming to have observed nearly 60% more starch than in controls in some experimental tubers. In comparison, Sweetlove *et al.* (1996a,b) found that while there was increased flux into starch metabolism, this was accompanied by an increased turnover rate, resulting in no overall increase in starch content. Stark *et al.* (1992) also expressed the *glg*C16 gene under the control of the 35s promoter of cauliflower mosaic virus, a highly expressing constitutive promoter. These transformants did not result in any viable transgenic plants, but the callus produced had considerably increased starch, accompanied by an increased number of starch granules, as observed under the light microscope. Another group has taken a more traditional approach to manipulation of this enzyme for increased starch content in maize. Giroux *et al.* (1996) exploited the maize *Dissociation* (*Ds*) system to target the maize gene for the large subunit of AGPase and create small insertion mutants within the gene. The *shrunken2-m1* mutation selected has a Ds transposable element already present within exon 16, an area known to be involved in allosteric regulation of this gene by phosphate. Revertants of this type of mutation are created by the excision of the *Ds* element, recreating the complete gene. However, inaccurate excision of the element can result in small in frame insertions in the gene creating an additional 1 or 2 amino acids in the protein sequence. Secondary mutants were created by reverting the *sh2-m1* mutant and sequencing the subsequent partial revertants to determine whether reversion had resulted in the production of in frame insertions, that is, insertions of 3 or 6bp that do not cause frameshift mutations, which inactivate the gene. One revertant in particular that had a 6bp insertion leading to an extra serine and tyrosine residue was noted to have an increase in seed weight of 11–18% over wild type, and this could in part be explained by an increase in the amount of starch. The authors concluded that seed weight increased as a result of increase of several seed components, not only starch, that the mutation resulted in a stronger sink, which, in turn, led to an increase in synthesis of other seed components.

3. Alteration of the amylose to amylopectin ratio has been a focus of much research, as starches with altered ratios have been shown to have many uses. This is partly because of waxy mutants, which produce starches with little or no amylose. These starches are used in both the food industry, where waxy starches have lower pasting temperatures and greater paste clarity than normal starches (Jane, 1997) and in non-food uses for, among other things, the manufacture of plastics (van Soest *et al.*, 1997), and paints (Taylor, 1997). Most waxy starches are created by

mutations in the GBSS gene, which have been selectively bred for in maize, and hence have been available for a number of years. Study of these mutants led to the initial isolation of the maize GBSS gene (Shure *et al.*, 1983; Klösgen *et al.*, 1986), which, in turn has led to the isolation of the equivalent gene in rice (Okagaki and Wessler, 1988) and potato (Visser *et al.*, 1989). This has resulted in a large number of antisense studies using the GBSS genes of several species to suppress GBSS activity and create low amylose and amylose free mutants (Visser *et al.*, 1991; Shimada *et al.*, 1993). In addition, the regulatory regions of these genes are being studied to gain an understanding of the pattern of gene expression of starch biosynthesis genes (e.g. Hirano *et al.*, 1995; Li *et al.*, 1995). Antisense experiments, especially in potato have enabled an increase in understanding of how amylose is laid down in the formation of the starch granule (Kuipers *et al.*, 1994), and also how dosage effect and amylose content are linked (Flipse *et al.*, 1996a). The advantage of using genetic manipulation for these studies is that it enables the effect of the gene suppression to be studied in a known genetic background, but eliminating the need for a lengthy breeding program with a large number of backcrosses. One crop that has no known naturally occurring phenotypic waxy mutant is wheat, where there is a strong interest in developing a low amylose variety. This would result in flour that would give extended shelf life for bread products. Waxy starches have also been shown to be superior for oriental noodles (Graybosch *et al.*, 1997). The hexaploid nature of wheat means that naturally occurring phenotypic null waxy mutants do not exist and efforts to develop waxy wheat starch have been laborious. Several groups have now developed waxy wheat lines utilizing breeding of partial waxy mutants (varieties mutant in one of the *waxy* alleles) (Nakamura *et al.*, 1995; Hoshino *et al.*, 1996) or random mutagenesis (Yasui *et al.*, 1997) and studies into the effects of the properties of the starches from these mutants are now in progress (Yasui *et al.*, 1996; Hayakawa *et al.*, 1997). The availability of a GBSS cDNA from wheat (Clark *et al.*, 1991) now means that it is theoretically possible to create a waxy wheat variety using antisense technology. This has the advantage that it would enable the waxy property to be inserted, in one generation, into a variety agronomically suitable for the required environment. Obtaining plants with a high amylose content is another goal, as high amylose starch has film formation properties particularly desirable for food uses such as fried food coating batters (Jane, 1997). High amylose starches in plastics also retain crystallinity during storage at high humidity in comparison to high amylopectin starches (van Soest *et al.*, 1997). Studies involving the expression of the GBSS gene in a normal amylose variety of rice resulted in sense suppression, suggesting that raising of amylose levels by the over-expression of the GBSS gene would not be possible due to natural feedback mechanisms (Itoh *et al.*, 1997). Maize in particular is a good source of high amylose starch, as the *ae* varieties, which are mutant in the BEII gene, have much reduced branching and result in a high apparent amylose content. No such mutants have been found in potato, although, as mentioned earlier, a BE gene in potato has been antisensed, producing an altered starch, but not one with any obvious increase in amylose (Flipse *et al.*, 1996d). Other high amylose mutants include the *ae* mutation in rice, which is thought to correspond to the BEIII gene (Mizuno *et al.*, 1993) and the original *r* mutant of pea, which encodes BEI (Bhattacharya *et*

al., 1990). It is possible that plant varieties with higher amylose levels could be engineered by suppressing more than one of the multiple BE genes within a system. Before the appropriate enzymes can be targeted, however, it is important to gain an understanding of the role of the various isoforms in the branching patterns of amylopectin. High amylose has also been found in the *sugary* mutants of maize (Shannon and Garwood, 1984), although the *sugary* mutants of rice actually have low or no detectable amylose (Kaushik and Khush, 1991). The *su-2* locus in maize is thought to encode a gene involved in gene regulation during endosperm biosynthesis (Nelson and Pan, 1995), as are the *sugary* genes from rice. This may explain the phenotypic differences between the two sets of mutants, as different regulatory genes may regulate different subsets of starch biosynthesis genes. An understanding of regulatory genes for starch biosynthesis and endosperm development may also lead to the development of varieties with altered starch. For example, while it may not be possible to over-express genes using transgenic technology directly (as studies on AGPase mentioned earlier have shown) manipulation of regulatory regions of genes may allow the normal feedback mechanisms to be overridden. Manipulation of regulatory genes may also allow for more than one gene to be influenced simultaneously, similar to the pleiotropic effects seen in the *sugary* mutants of maize and rice. For example, while it may be difficult to switch off all known branching enzymes, it may be possible to reduce branching extremely efficiently by antisensing a key regulatory gene. Drawbacks to this sort of technique at the moment include our lack of understanding of many of these genes and the fact that many of those that have been studied do seem to regulate quite a large number of quite different genes. For example, the *Flo-2* locus of rice is thought to control expression of BEs, DBE GBSS and AGPase (Kawasaki *et al.*, 1996).

4. A continuation of the concept of alteration of amylose:amylopectin ratio is to manipulate the levels of branching within the amylopectin, without actually altering its percentage of the total starch. This is particularly important as the level of branching and the length of amylopectin chains are both thought to be key components in determining physical properties of the starch. For example, an increase in the shorter B chains in starches would increase resistance to shear thinning and increase the viscosity of starch pastes, which would prevent the need for chemical crosslinking. Alternatively, shortening of A chains could be used to convert an enzyme resistant starch such as banana to a more easily digestible form. Altered branching properties would also affect the quality of plastics produced from starch. Many of the experiments into manipulating branching levels of starch have involved the over-expression of heterologous enzymes from *E. coli* and resulted in a highly branched, unstructured amylopectin, similar in structure to glycogen. While this would be expected from the expression of *E. coli* glycogen branching enzyme, which has been expressed in amylose free potato (Kortstee *et al.*, 1996), more surprising is that expression of the *E. coli* glycogen synthase enzyme also resulted in production of a highly branched starch (Shewmaker *et al.*, 1994). This is only one of many experiments that suggest that our current theories as to the role of various enzymes in starch structure are too simplified and that the reality involves complex interactions between the various synthases and branching enzymes. This is confirmed by reciprocal experiments

where maize BEs I and II have been used to complement branching enzyme mutations in *E. coli*. Despite the enzyme activity being shown to be similar to that of maize purified enzyme, the resultant polysaccharide has been shown to be highly branched and more analogous to glycogen than starch. Despite this, the two different enzymes did produce slightly different chain lengths, with BEI giving and average chain length of 14, while BEII gave an average chain length of 16 (Guan *et al.,* 1995). This is in comparison to an average chain length of 22–24 in normal maize amylopectin, and 10 for *E. coli*. Surprisingly, the result of expressing both BEs together resulted in an average chain length of 12 (Guan *et al.,* 1995). The role of the various starch synthases in controlling branching in *Chlamydomonas* has recently been studied by Buléon *et al.* (1997). This group used mutants for GBSS, SSSI or SSSII and compared the starch structure with that of the wild type. What they found was that deficiency in any one of the SSs resulted in a change in the amylopectin structure, and that each SS appeared to be involved with branching at a different level. Deficiency of GBSS resulted in not only loss of amylose, but loss of a high molecular weight portion of the amylopectin (Delrue *et al.*, 1992). Deficiency in SSSII was found to show a decrease in intermediate sized glucans, causing a B type diffraction pattern and a general loss of crystallinity (Fontaine *et al.,* 1993). Mutants deficient in both GBSS and SSSII, and hence completely dependant on SSSI for starch synthesis, were found to have only a very small amount of granular starch that was highly branched, similar to phytoglycogen (Maddelein *et al.,* 1994). Thus, these authors concluded that all the SS enzymes have an individual role in determining the structure of amylopectin either by synthesizing particular length chains, or by interaction with particular BEs. The problem with such experiments so far, is that while giving some clues into the complex nature of the regulation of branching length and number, they have not enabled any specific manipulation of branch length or number of the type that would be needed for industrial uses such as plastic manufacture.

5. Starch granule size is one of the main variables between starches of different botanical origin, and a key factor in choice of starch type for particular applications. Maximum viscosity of starch solutions is thought to be reached at maximum granule swelling during simultaneous heating and shearing, thus viscosity will be related to both size of the granule and accessibility of water to the crystalline structure. This in turn affects factors such as thickening properties and retrogradation after subsequent cooling and heating, particularly relevant with the increase in food products such as frozen meals (Lillford and Morrison, 1997). Starch in its granular form is used in applications such as dusting agents for confectionery and face powders. Applications where granule size is a key factor in choice of botanical origin include carbonless copy paper, which uses the large, smooth granules of wheat starch (Nachtergaele and Van Nuffel, 1989), and degradable plastic film, where granule size affects tensile strength and film thickness (Lim *et al.*, 1992). There is also interest in developing the use of small starch granules (e.g. rice, cassava and waxy maize) as fat mimetics, as a starch granule with a diameter of 2 microns or less will give products a similar mouthfeel and taste to fat (Daniel and Whistler, 1990). Different granule types also exhibit different digestibilities depending on their crystallinity types, with potato and banana starches (B and C type crystallinity) having poor digestibility (Jane, 1997).

Despite the importance of the starch granule size and structure with respect to functionality, very little is understood about the factors influencing this. Some mutants, notably the *sugary* (*su1* and 2) mutants of maize and the rugosus (*r, rug* and *rb*) mutants of pea (Lloyd *et al.*, 1996), are known to reduce granule size. However, in all cases, this seems to be a by product of a reduction in the total amount of starch produced. It is likely that more subtle changes would need to be studied to establish genetic factors involved in starch granule morphology, as the difference in starch granule size in varieties of maize are strongly indicative of alleles that play a role in this (Campbell *et al.*, 1996), although it is highly likely that environment is also important. The factors behind the bimodal distribution of starch granule size in cereals such as wheat and barley are another area where much research is needed as it has been shown for barley that not only do the size of the A and B granules vary with variety, but the ratio of B to A granules can vary from 9.6 to 16.8 (Oliveira *et al.*, 1994). Granule type ratio would be an important factor to control as, for example, a wheat that produced only A granules would be useful for production of carbonless paper as the A granules would not have to be separated from the unwanted B granules. The expression of the *E. coli glg*B gene for glycogen synthase mentioned earlier (Shewmaker *et al.*, 1994) as well as resulting in a starch with a much more branched structure, was noticed to have resulted in a marked increase (up to 80%) in small, round starch granules of less than 10 μm, similar to cereal starches. As this was linked with an increase in amylopectin content, it was speculated by the authors that granule size and amylopectin levels may in some ways be linked. However, the presence of a high amount of sucrose in these tubers may also suggest that this effect was, similar to the pea mutants of Lloyd *et al.* (1996), merely due to limited overall starch biosynthesis.

6. Other constituents such as lipid, protein and phosphate are also part of the native starch structure within the plant, and these are also factors that can influence the properties of the starch. For example, reduction in lipid, especially phospholipid can result in increased clarity of starch pastes, one reason why waxy starches are often used, as they have particularly low levels of phospholipid. Lipid interactions with amylose are also thought to be a factor in determining physical properties such as swelling power and viscosity (Visser and Jacobsen, 1993). Protein content, especially around the starch granule, is thought to play a role in controlling granule swelling during processing (Lillford and Morrison, 1997) and control of this, once it is better understood, may also result in starches with better processing qualities. Protein content is also thought to play a role in starch flavour in food applications, and for non-food uses, protein removal is desirable, if somewhat difficult in the case of small granule sized starches. A large proportion of these proteins in cereals are storage proteins such as glutelins and gliadins for which genes have been cloned. While some antisensing of such genes has taken place in rice, to reduce allergic reactions caused by these proteins, this did not have any effect on the overall amount of protein produced in the seed (Nakamura and Matsuda, 1996; Tada *et al.*, 1996). This suggests that a large number of genes would have to be antisensed before significant protein reduction could be obtained. As large gene families encode most seed storage proteins, antisensing all these genes would be so complicated as to be impractical (Nakase *et al.*, 1996). Some

mutants have been shown to be deficient in a group of proteins, e.g. the *Opaque-2* gene of maize encodes a transcriptional activator and mutants in this gene have been shown to be deficient in certain zein storage proteins (Hartings *et al.*, 1989). However, such mutants also tend to have altered starch, so at present it seems unlikely that protein content could be reduced by any manipulation of regulatory proteins until the mode of action and specificity of these proteins is understood. Other factors are often produced by chemical modifications of processed starch, but have the potential to become 'value added' properties of native starch with such starches having the advantage of being more environmentally friendly to produce. Processing of starch under harsh conditions such as low pH and high temperature means that starch is often crosslinked prior to processing to prevent loss of viscosity. Starch that has been genetically modified so that it does not require chemical crosslinking prior to processing would be valuable, not only to remove the need for the chemical process, but because only some of the available methods for crosslinking starch chemically have been approved for food use, and use of, for example, crosslinked hydroxypropylate starch for frozen food applications is becoming desirable (Jane, 1997; Champagne, 1996). Phosphorylation is a modification known to have an affect on rheological properties of starch, and phosphorylation of potato starch is used to control function and appearance for food uses such as salad dressings (Poulsen *et al.*, 1997). As a certain amount of phosphorylation of amylopectin occurs naturally, and amounts vary according to potato type, it does not seem inconceivable that higher levels of natural phosphorylation could be achieved either through selective breeding for natural mutants, or by genetic modification, if the enzyme responsible for phosphorylation could be identified. Shewmaker *et al.* (1994), in the previously discussed expression of *E. coli glg*B in potato, noted that, in addition to changes in the starch granules and fine structure, overall phosphate content was significantly lower than in normal potato starch. One possibility is that, as phosphate tends to be associated with longer chains, this reduction was simply a result of the shorter chain structure of this starch. However, the authors comment that phosphate incorporation is poorly understood, and other factors, linked to the normal starch synthases in potato may be important.

7. There is also the possibility that the addition of heterologous enzymes into the plant genome could be used to make specialist carbohydrate products utilizing starch as a substrate. Cyclodextrins are cyclic oligosaccharides of 6,7 or 8 glucose molecules which can be synthesized by bacterial cyclodextrin glycosyltransferases (CGT), using starch as a substrate. Cyclodextrins are much in demand as pharmaceutical delivery systems and flavour and odour enhancement and removal systems as they have an apolar cavity which enables them to form a complex with a hydrophobic 'guest' molecule. Such complexes enable the guest molecule to have increased solubility and prolonged stability. Currently, cyclodextrins are synthesized in batch fermentors using hydrolyzed starch as a substrate, but this method is expensive for large scale use. Synthesis of cyclodextrins in plants has potential in that the one enzyme, CGT, required for conversion of starch to cyclodextrin is available. Oakes *et al.* (1991) used a CGT cloned from *Klebsiella pneumoniae* under the control of the patatin promoter and transformed this into potatoes. Although the amount of mRNA produced in the resultant transformants

was low (estimated at 0.00001–0.0001% of total mRNA) both 6 ring, and in some cases 7 ring cyclodextrins were detected in the transformed tubers at levels corresponding to conversion of 0.001–0.01% of starch. Although such levels are low, expression has the potential to be increased by uses of other bacterial CGT genes, promoters and alteration of factors such as codon usage, which may improve expression of a bacterial enzyme in plants. Understanding the factors which determine specificity of hydrolysis and synthesis of glycosidic bonds in the synthesis of carbohydrate generally in plants could also be the basis of production 'designer' carbohydrates using plants as molecular factories.

Potential new targets for starch manipulation

Future possibilities for manipulation of starch, some of which have already been considered above, are numerous in the long term, but many depend on further research into the mechanisms of starch biosynthesis, and the physical properties of altered starch structures. One area, which has been studied extensively at the physical level, is the structure of the starch granule. Current theories are based on the blocklet structure, which explains both molecular and electron microscope observations (*Figure 1*). While the dimensions of the amorphous and crystalline components of a single blocklet can vary with factors such as amylose content, the length of a single blocklet has been found to be constant, irrespective of botanical origin and mutant phenotypes, at 9 nm. Alteration of some aspect of starch metabolism that would alter this parameter would give an interesting insight into the more constant aspects of starch metabolism, as well as possibly conferring novel properties on such a starch granule. Another poorly understood area is the actual biogenesis of the starch granule. While many plants (e.g. potato) contain only one starch granule in a single amyloplast, others such as rice have amyloplasts with many starch granules. There is obvious similarity between the amyloplast and the chloroplast, and gene targeting sequences seem to work equally well for either organelle, with the chloroplast ribulose 1,5-bisphosphate carboxylase targeting sequence from *Arabidopsis* being able to target the potato amyloplast (Stark *et al.*, 1992) and the maize GBSS targeting sequence also mediating protein transport, at least *in vitro*, to the chloroplast (Klösgen *et al.*, 1989). This is not surprising, in that chloroplasts contain starch granules; however, this leaves the questions as to what the main signal initiating storage starch granule biosynthesis might be. Identification of a starch priming molecule, analogous to glycogenin in mammalian systems, could give some major ideas as to the biogenesis of the starch granule, and manipulation of the levels and sequence of such a molecule may enable control of either the number or size of starch granules in a plant. This would be particularly useful given the current use for small starch granules in both food and non-food applications, as then granule size in a high starch yielding crop such as potato could be controlled. Studies on the current candidate, amylogenin, which at least appears to perform the biochemical reaction required of a starch primer, may improve our knowledge in this area. As both wheat and barley contain starch granules of two sizes, understanding of ways in which one or other type could be selected in these crops would be extremely useful, as wheat A type granules are particularly good quality for the manufacture of carbonless copy paper, while the B type granules have potential to be used as fat mimetics. At the moment, the granule types have to be

separated post harvest, which adds cost to the product, or processed mechanically to achieve a smaller granule size (Jane *et al.*, 1992).

Further understanding of the fine structure of branching levels and how these may be manipulated accurately is also required, as current manipulations in this area, while being interesting, have mainly resulted in loss of the regular structure of starch and production of phytoglycogen. One way in which some understanding may be achieved is by comparison of the branching levels of different starches and trying to relate these to differences in enzymes involved in the synthesis of these starches. One possibility would be to express enzymes from one botanical origin in an alternative host, and see whether this affects starch structure at the molecular level. Given the current theories regarding regulation of branching being controlled by DBE (Ball *et al.*, 1996), this enzyme would be a likely candidate for such studies. Further study is also needed into the functionality of such starches regarding how number and length of branches correlate to factors such as glass transition temperature and gelling and pasting qualities in engineered starches. It has also been proposed (Lillford and Morrison, 1997) that as lipid complexed within amylose is known to play a role in the reduction of starch retrogradation, that there is a possibility for manipulation of lipid levels to reduce retrogradation in high amylose starches. However, until the manner of starch/lipid interaction is better understood, this remains a goal for the future.

Given the variation in climate across the world, there is also a need to understand the mechanisms of starch biosynthesis. This would be useful not only in the major crops such as maize, potato and rice, but also in wheat, which is currently lagging behind its other cereal counterparts, mainly due to the hexaploid nature of its genetics, and barley, which has the advantage of being a particularly robust crop in colder climates such as Scandinavia. In addition to being able to manipulate crops suited to particular climates, starch structure is known to vary within a particular geographical area depending on climatic conditions within a growing season. This is particularly well documented for amylose content in rice, where one allele of the GBSS gene in particular has been shown to be particularly susceptible to temperature (Sano *et al.*, 1985). This seasonal variation of crop quality can interfere with product quality, for example, the processing of rice to produce crisped rice breakfast cereal is dependent on an ideal level of amylose in the rice to produce a product of optimum quality. Studies into the understanding of the causes of such seasonal variation, and how this may be overcome are an area where research is needed and would be useful not only in maintaining crop and product quality, but would also provide useful information regarding starch biosynthesis of gene regulation, and possibly more widespread information regarding plant gene expression.

Conclusion

In conclusion, in recent years there have been huge advances in our knowledge regarding the fine structure of starch, the enzymes involved in the biosynthesis of this fine structure and how this relates to product quality. There are still many gaps in this knowledge relating to how minor required changes may be controlled to produce starches for specific industrial uses, and also how gross changes in starch granule morphology may be effected, but our knowledge is expanding constantly. There are also more practical hurdles to be overcome before genetically engineered starches

become common in the market place, not least being acceptance of the consumer, who in Europe, at least, is being slow to accept the products of plant genetic engineering on the supermarket shelves. Such problems have not however, occurred with starches from plants obtained through selective breeding of mutants. There is also the question of such plants (both mutants and transformed) producing high enough yields of required starches to make farmers consider their use economic. It is likely, though, that the needs of the marketplace will drive research into these areas forward and it will not be long before the first fruits of molecular genetic engineering are in the market place.

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